Ouabain downregulates Mcl-1 and sensitizes lung cancer cells to TRAIL-induced apoptosis

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THE APOPTOSIS OF CANCER CELLS in response to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a prerequisite for cancer progression, and TRAIL resistance is prevalent in lung cancer. Ouabain, a recently identified human hormone, has shown therapeutic promise by potentiating the apoptotic response of metastatic lung cancer cells to TRAIL. Nontoxic concentrations of ouabain are shown to increase caspase-3 activation, poly(ADP-ribose) polymerase (PARP) cleavage, and apoptosis of H292 cells in response to TRAIL. While ouabain had a minimal effect on c-FLIP, Bcl-2, and Bax levels, we show that it possesses an ability to downregulate the antiapoptotic Mcl-1 protein. The present study also reveals that the sensitizing effect of ouabain is associated with its ability to generate reactive oxygen species (ROS), and hydrogen peroxide is identified as the principle ROS triggering proteasomal Mcl-1 degradation. In summary, our results indicate a novel mechanism of action for ouabain in TRAIL-mediated cancer cell death through Mcl-1 downregulation, thereby providing new insight into a potential lung cancer treatment as well as a better understanding of the physiological activity of ouabain.

Ouabain; lung cancer; TRAIL; Mcl-1

Ouabain is a cardiac steroid that functions as a specific inhibitor of the Na^+-K^+-ATPase and is used in clinical practice for the treatment of several heart-related maladies, such as atrial fibrillation and heart failure (17, 32). As ouabain has been found in human plasma (6, 9, 19–20, 25–28) and identified as a human hormone (21, 26), information regarding other activities of this compound is of great interest and will benefit both physiological and pathological knowledge. Although some studies have reported the apoptotic induction property of ouabain in cancer cells (4, 11, 23, 35), the molecular basis of this induction and the precise role of this compound in TRAIL-mediated cancer cell death are not known. Therefore, in the present study, we demonstrate that administration of ouabain significantly accentuates TRAIL-induced apoptosis in lung cancer cells. The proposed mechanism of sensitization involves the ability of the compound to induce reactive oxygen species (ROS) generation and Mcl-1 downregulation. Our findings reveal the existence of a novel mechanism of action for ouabain in the regulation of Mcl-1 degradation and TRAIL sensitization, which could be important in the understanding of physiological and pathological mechanisms and in the development of cancer therapy.

MATERIALS AND METHODS

Cells and reagents. NCI-H292 and H460 cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI 1640 containing 10% fetal bovine serum, 2 mM l-glutamine, and 100 U/ml penicillin/streptomycin in a 5% CO_2 environment at 37°C. Cell permeable glutathione monoethyl ester (GSH), catalase, Mn(III)tetrakis(4-benzoic acid)porphyrin chloride (MnTBAP), MG123, concanamycin A, ouabain, dichlorofluorescein diacetate (DCFH_2-DA), and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma Chemical (St. Louis, MO). Propidium iodide (PI) and Hoechst 33342 were obtained from Molecular Probes (Eugene, OR). Antibodies for Mcl-1, Bcl-2, Bax, c-FLIP, caspase-3, and poly(ADP-ribose) polymerase (PARP) as well as peroxidase-conjugated secondary antibodies were obtained from Cell Signaling Technology (Beverly, MA). Other reagents were obtained from Sigma Chemical (St. Louis, MO) or other sources.

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obtained from Cell Signaling (Danvers, MA). The β-actin antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The transfecting agent Lipofectamine 2000 was obtained from Invitrogen (Carlsbad, CA).

**Plasmid and transfection.** The Mcl-1 expression plasmid (pcDNA3.1-hMcl-1) was obtained from Addgene (Cambridge, MA). The Mcl-1 knockdown plasmid (shMcl-1) and control plasmid were obtained from Santa Cruz Biotechnology. Stable transfection of Mcl-1 was established by culturing cells in a six-well plate until they reached 60–70% confluence. Fifteen microliters of Lipofectamine 2000 reagent and 2 μg of Mcl-1 shMcl-1, or control plasmid were used to transfect the cells in the absence of serum. After 12 h, the medium was replaced with culture medium containing 10% fetal bovine serum. Approximately 2 days after the beginning of transfection, the cells were digested with 0.025% trypsin, plated onto 75-cm² culture flasks, and cultured for 30 days with antibiotic selection. The stable transfectants were pooled, and the expression of Mcl-1 protein in the transfectants was confirmed by Western blotting. The cells were cultured in G418-free RPMI 1640 medium for at least two passages before use in each experiment.

**Cell viability and apoptosis assays.** Cell viability was evaluated using the MTT assay. After the indicated treatments, cells were incubated with 500 μg/ml of MTT at 37°C for 4 h. An intensity reading of the MTT product was measured at 550 nm using a microplate reader, and the percentage of viable cells was calculated relative to control cells. Apoptosis was determined by Hoechst 33342/PI staining and DNA content analysis. Cells were washed and incubated with 10 μg/ml Hoechst 33342 and 5 μg/ml PI for 30 min. Nuclei condensation and DNA fragmentation of apoptotic cells and PI-positive necrotic cells were visualized and scored by fluorescence microscopy (Olympus IX51 with DP70). In the case of sub-G0 DNA content analysis, after the specified treatment, cells were trypsinized, washed with PBS, and fixed in 70% ethanol at 37°C for 3 h. After being washed with PBS, cells were incubated in PI solution containing 0.1% Triton-X, 1 μg/ml RNase, and 1 mg/ml PI at room temperature for 30 min. DNA content was analyzed using flow cytometry (FACSOr; Becton Dickinson, Rutherford, NJ).

**ROS detection.** Intracellular ROS were determined using DCFH2-DA as a specific ROS probe. After specific treatments, cells were incubated with 10 μM of DCFH2-DA for 30 min at 37°C, after which they were washed, trypsinized, resuspended in PBS, and immediately analyzed for fluorescence intensity using a microplate reader.

**Western blotting.** Cells were incubated in lysis buffer containing 20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 150 mM sodium chloride, 10% glycerol, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 100 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail (Roche Molecular Biochemicals) for 40 min on ice. The cell lysates were collected, and the protein content was determined using the Bradford method (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein from each sample (60 μg) were denatured by heating at 95°C for 5 min with Laemmli loading buffer and subsequently loaded onto a 10% SDS-PAGE. After separation, proteins were transferred onto 0.45 μM nitrocellulose membranes (Bio-Rad). The transferred membranes were blocked for 1 h in 5% nonfat dry milk in TBS-T (25 mM Tris-HCl pH 7.5, 125 mM NaCl, and 0.05% Tween 20) and incubated with the appropriate primary antibodies at 4°C overnight. Membranes were washed twice with TBS-T for 10 min and incubated with horseradish peroxidase-coupled isotype-specific secondary antibodies for 1 h at room temperature. The immune complexes were detected by enhancement with a chemiluminescent substrate (Supersignal West Pico; Pierce, Rockfore, IL) and quantified using analyst/PC densitometry software (Bio-Rad).

**Immunoprecipitation.** Cells were washed after treatments and lysed in lysis buffer at 4°C for 20 min. After centrifugation at 14,000 g for 15 min at 4°C, the supernatants were collected and assayed for protein content. Cleared lysates were normalized, and 60 μg of each protein were incubated with an anti-Mcl-1 antibody conjugated to protein G plus-agarose beads (Santa Cruz) for 4 h at 4°C. The precipitated immune complexes were washed five times with ice-cold lysis buffer, resuspended in 2× Laemmi sample buffer, and boiled at 95°C for 5 min. Immune complexes were separated by 10% SDS-PAGE and analyzed by Western blotting using an anti-ubiquitin antibody.

**Statistical analysis.** Mean densitometry data from independent experiments were normalized to results of the control cells. The data are presented as the means ± SD from three or more independent experiments. Statistical differences between the means were determined using an ANOVA and post hoc test at a significance level of \( P < 0.05 \).

**RESULTS**

**TRAIL-mediated apoptosis in H292 lung cancer cells.** TRAIL-induced cell death is accepted as an important inhibitory mechanism against cancer progression in most cancers. To explore the sensitizing effect of ouabain, prerequisite information about TRAIL-mediated death characteristics is crucial. Human lung carcinoma H292 cells were treated with various doses of TRAIL (0–80 ng/ml) for 14 h, and apoptosis and cell viability were analyzed using Hoechst 33342 staining, sub-G0 DNA content analysis, and MTT assays. Figure 1A shows that TRAIL treatment caused a concentration-dependent increase in apoptosis that was detected in ~25 and 50% of cells in response to 20 and 80 ng/ml of TRAIL, respectively. DNA content analysis showed an increase in the population of cells in the sub-G0 phase in response to TRAIL treatment (20–40 ng/ml), whereas the sub-G0 phase was not altered in the 10 ng/ml TRAIL-treated cells compared with control nontreated cells (Fig. 1B). Analysis of cell viability further showed that TRAIL caused a decrease in cell survival in a dose-dependent fashion (Fig. 1C). This result suggests that the mode of cell death in TRAIL-treated cells was mainly apoptosis.

Western blot analyses of cells in response to TRAIL strongly supported apoptotic pathway induction in such cells. An increase in activated caspase-3 and the cleavage of PARP in response to TRAIL at concentrations of 20 and 40 ng/ml was consistent with the increase in apoptotic cells presented in Fig. 1, A and B (Fig. 1D).

**Oubain sensitizes cells to TRAIL-mediated apoptosis.** Ouabain-mediated cancer cell death has been reported in some cancers (4, 11, 23, 35), and we have found that ouabain at concentrations of 5–10 nM causes a significant increase in apoptosis compared with nontreated control cells (Fig. 2A). Our analysis of sub-G0 DNA content supported that apoptosis was the primary mode of cell death (Fig. 2B), and the accompanying viability assay indicated that at concentrations >5 nM ouabain induces cytotoxic effects in H292 cells (Fig. 2C). Figure 2D further confirms that the reduction of cell viability as well as the presence of apoptosis in ouabain-treated cells is elicited through a caspase-3-dependent mechanism that could be detected at concentrations >5 nM. Interestingly, ouabain at concentrations of 0–0.5 nM, which are comparable to the level of ouabain in human plasma (9, 20, 29), had only a minimal effect on cell viability.

The effects of nontoxic and physiological concentrations of ouabain on TRAIL-induced apoptosis were further evaluated. H292 cells were incubated with various concentrations of TRAIL (0–40 ng/ml) in the presence or absence of 0.5 nM ouabain. Cell viability and apoptosis were evaluated as described after 14 h. Figure 2E shows that the administration of
ouabain significantly enhanced the apoptotic response of H292 cells to TRAIL. Sub-G0 DNA content analysis also supported the apoptosis sensitization effect of ouabain in TRAIL-treated cells (data not shown). Furthermore, viability assays revealed the same trend as mentioned above, with the addition of ouabain sensitizing the cells to TRAIL-induced toxicity in which ~40% of cells remained viable in the presence of 0.5 nM ouabain compared with 80–90% viability in the groups receiving TRAIL treatment alone (Fig. 2F). Concurrently, Western blot analyses also exhibited that caspase-3 and PARP were remarkably activated in response to TRAIL in the presence of ouabain compared with only TRAIL treatment (Fig. 2G). These results support the potential role of ouabain in the enhancement of apoptosis.
Mcl-1 downregulation is crucial for the sensitizing effect of ouabain. Recent evidence has indicated that Mcl-1 plays an important role in attenuating TRAIL-mediated cancer cell death (10, 14, 30). To provide supporting evidence for the role of Mcl-1 in the TRAIL-mediated death of H292 cells, we evaluated the expression profile of Mcl-1 after cellular exposure to TRAIL. Cells were incubated with 0–40 ng/ml of TRAIL and analyzed for Mcl-1 expression by Western blotting. Figure 3A shows that such treatment caused a gradual decrease in Mcl-1 protein expression in a dose-dependent manner. However, the noncytotoxic concentration of TRAIL (10 ng/ml) caused no change in Mcl-1 protein level in these
cells. Mcl-1 modulation was also evaluated in response to ouabain treatment. These results indicated that ouabain treatment caused a significant decrease of Mcl-1 protein. It is noteworthy that even at the nontoxic concentration ouabain significantly suppressed Mcl-1 protein levels. The above findings suggest that Mcl-1 downregulation might be a possible mechanism in the ouabain-sensitized TRAIL response in these lung cancer cells.

To provide support to the data mentioned above, other proteins that have been identified as key players in the regulation of TRAIL-mediated death, either as inhibitors or promoters, were detected in the cells treated with ouabain and TRAIL. Figure 3B shows that the expression levels of the antiapoptotic Bcl-2 and c-FLIP proteins and the proapoptotic Bax protein were not altered in response to ouabain and TRAIL treatments, while the reduction of Mcl-1 was consistently detected. These data provide evidence that ouabain enhances the cellular response to TRAIL via a Mcl-1 downregulating pathway.

**Ouabain enhances TRAIL-induced apoptosis through a hydrogen peroxide-dependent pathway.** It has been demonstrated in several cell models that ouabain treatment results in ROS induction (11, 31). We analyzed cellular ROS in cells exposed to ouabain and TRAIL using a fluorescence microplate reader with DCFH-DA as a fluorescent probe. Figure 4A shows that ouabain, but not TRAIL, induced an increase in cellular ROS in a dose-dependent manner. A significant increase of the cellular ROS level was observed as early as 1 h after treatment and peaked at 2 h (data not shown).

To identify the specific ROS responsible for the sensitizing activity of ouabain, cells were pretreated with various known ROS scavengers, including GSH, MnTBAP (superoxide anion scavenger), catalase (hydrogen peroxide scavenger), and deferoxamine (hydroxyl radical inhibitor), followed by treatment with TRAIL in the presence of ouabain. Intracellular ROS levels were determined using a fluorescence microplate reader with DCFH-DA as an oxidative probe. Results showed that treatment of the cells with GSH and catalase inhibited the cellular fluorescence intensity, whereas deferoxamine and MnTBAP had no inhibitory effect (Fig. 4B). These results suggest that hydrogen peroxide is the primary ROS generated under these conditions.

We next evaluated the role of ROS on ouabain’s sensitizing activity; cells were pretreated with the mentioned ROS scavengers and treated with TRAIL and ouabain. Cell viability and apoptosis were determined after 14 h of treatment. Results indicated that in response to TRAIL and ouabain cell apoptosis significantly increased (Fig. 4C) in accordance with the decrease in cell viability (Fig. 4D). In the presence of the antioxidants GSH and catalase, the cytotoxic effect of TRAIL and ouabain was significantly suppressed, whereas MnTBAP
and deferoxamine exhibited insignificant effects. Together, these results strongly support the earlier findings that hydrogen peroxide is a key ROS involved in the TRAIL-sensitizing mechanism of ouabain.

**Oubain enhances the TRAIL-mediated downregulation of Mcl-1 through a hydrogen peroxide-dependent mechanism.** Oubain was earlier shown to promote TRAIL-induced apoptosis through the downregulation of Mcl-1, and this apoptosis-sensitizing effect was mediated by hydrogen peroxide generation. To investigate the linkage between these two events, H292 cells were pretreated with the ROS scavengers described above and treated with TRAIL in the presence of ouabain. Western blot analysis revealed that the reduction of Mcl-1 expression in response to ouabain and TRAIL treatments was
significantly inhibited in cells pretreated with glutathione and catalase (Fig. 4, E and F), whereas protective effects of other antioxidants were not observed. These findings support that hydrogen peroxide is a key mediator responsible for Mcl-1 downregulation and, hence, the apoptosis-sensitizing effect of ouabain.

**Ouabain enhances Mcl-1 degradation through the ubiquitin-proteasomal pathway.** Having shown that Mcl-1 downregulation in the ouabain-treated cells is involved with TRAIL sensitization, the present study further investigated the mechanism by which ouabain treatment induced the decrease in Mcl-1 levels. It is known that the Mcl-1 protein has a short half-life and that its expression level is controlled by proteasomal degradation (22). To test whether the downregulation of Mcl-1 in cells treated with TRAIL and ouabain was consistent in these contexts, cells were treated with TRAIL and ouabain in the presence of the specific proteasomal inhibitor MG132. Figure 5A indicates that the decrease in Mcl-1 levels was consequently inhibited in the proteasome-suppressed cells, revealing the involvement of proteasomal degradation in response to TRAIL and ouabain treatment. In addition, the lysosomal inhibitor concanamycin A was also used in this experiment, and the results demonstrated that the lysosomal pathway had no effect on Mcl-1 downregulation under these conditions. Because ubiquitination is a critical prerequisite process before proteasomal cleavage, Mcl-1-ubiquitin complexes in response to TRAIL and ouabain treatment were detected by immunoprecipitation. Figure 5B shows that the cells treated with TRAIL and ouabain exhibited a significant increase in Mcl-1-ubiquitin complexes, revealing that the treatment increased the degradation rate of Mcl-1.

**Ouabain enhances TRAIL-induced apoptosis in Mcl-1-overexpressed cells.** Amplification or overexpression of Mcl-1 has been tightly related to TRAIL resistance in many cancer cells (13, 30, 34). To test whether Mcl-1 expression might determine cell death after TRAIL treatment, we stably transfected the cells with Mcl-1, shRNA Mcl-1, or control plasmid and evaluated the effects on TRAIL-induced apoptosis. Because Western blotting revealed the substantial modulation of Mcl-1 as indicated in Fig. 6A, cells were cultured, exposed to various doses of TRAIL (0–40 ng/ml) for 14 h, and assayed for apoptosis. The Mcl-1-transfected cells exhibited less apoptosis than the control cells when treated with TRAIL, whereas shRNA-Mcl-1-transfected cells showed a greater number of apoptotic cells (Fig. 6B). These results indicated that Mcl-1 plays a negative regulatory role in the TRAIL-mediated death of these cells.

Further, the effect of ouabain on these Mcl-1 transfectants was investigated. Our results indicated that ouabain administration significantly enhanced the apoptotic response of all Mcl-1-modulated cells to TRAIL (Fig. 6B). Notably, the degree of ouabain sensitization correlated well with the levels of basal Mcl-1 in these cells. The shMcl-1 cells possess the lowest level of Mcl-1 and displayed the most ouabain sensitization, while Mcl-1-overexpressing cells contained the highest level of Mcl-1 and showed less response to ouabain. Mcl-1 expression levels were also decreased in response to the presence of ouabain pretreatment in Mcl-1 transfectants incubated with TRAIL (0–40 ng/ml; Fig. 6C).

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**Ouabain sensitizes H460 human lung cancer cells to TRAIL-induced apoptosis.** To test whether ouabain could play a sensitizing role in the TRAIL-induced apoptosis of other lung cancer cells, the H460 human lung cancer cell line was stably transfected with Mcl-1, shMcl-1, or control plasmids as described in MATERIALS AND METHODS. After selection, the cells were analyzed for Mcl-1 expression and apoptotic responses to TRAIL in the presence or absence of ouabain. Figure 7, A and B, shows that the Mcl-1-transfected H460 cells exhibiting the highest level of Mcl-1 protein expressed the highest TRAIL resistance, whereas the shMcl-1-transfected cells exhibited the lowest Mcl-1 level and degree of TRAIL resistance. In ouabain-treated groups, a significant accentuated effect on TRAIL-induced apoptosis was observed in all tested cells (Fig. 7B). Ouabain was also able to decrease Mcl-1 expression levels in TRAIL-resistant, Mcl-1-overexpressing cells (Fig. 7C). The above results are consistent with the earlier findings in H292...
cells and indicate the general role of Mcl-1 and ouabain in TRAIL sensitization in lung cancer cells.

**DISCUSSION**

TRAIL-induced apoptosis in cancerous cells plays an important role in controlling carcinogenesis and the progression of such disease. Resistance of the cancer cells by either the induction of antiapoptotic proteins or defects in apoptosis pathways often allows the cancer to progress. Even though previous studies have demonstrated that high concentrations of ouabain could induce apoptosis in some cancer cells (4, 11, 23, 35), the effects of noncytotoxic concentrations of such compounds, which may be linked to biological functions, are unknown. Initiating from the discovery of endogenous ouabain and related compounds in human plasma and tissue, the roles of ouabain in the regulation of physiological functions as well as in pathological conditions have been intensively investigated. Indeed, evidence has indicated that the plasma ouabain concentration increases in certain pathological conditions including hypertension and cardiac and renal failure (4, 16); however, the function of ouabain in immune-related reaction processes is still largely unknown. Our study suggests for the first time that endogenous ouabain might play a role in rendering an immune defense against cancers. We found that ouabain at a concentration of 0.5 ng/ml (~0.85 nM), which is comparable to the human plasma concentrations (0.16–0.77 nM) (9), played an important role in sensitizing lung cancer cells to TRAIL-induced apoptosis.

A recent study presented that ouabain regulates the interaction of Src and the Na⁺-K⁺-ATPase, which results in Src activation and consequently enhances cell growth, as well as increases intracellular calcium and promotes myocyte contraction (16, 31, 37, 30). Conversely, our data demonstrated that the number of apoptotic cells in response to a Src inhibitor in TRAIL and ouabain cotreated cells was not significantly altered compared with TRAIL and ouabain cotreated control cells (data not shown). It was reported that the effect of the Na⁺-K⁺-ATPase was cell specific due to the expression of various enzyme subunits in different cell types (5, 16). Although the Na⁺-K⁺-ATPase-Src pathway was activated in response to ouabain treatment, the dominant mechanism of “ouabain-enhanced TRAIL-induced apoptosis” may not be involved.

Previous studies have revealed that ouabain induces the generation of ROS and apoptosis in human neuroblastoma cells.
Our results strongly support these notions, as the Mcl-1-overexpressing H292 cells exhibited dramatic resistance to TRAIL-induced cell death while Mcl-1 knockdown cells showed the opposite effect (Fig. 6B). Such an effect of Mcl-1 was consistent in the H460 human lung cancer cell model (Fig. 7B). In addition, we found that ouabain enhanced TRAIL-mediated cell death in both H292 and H460 cells.

In conclusion, our current study reveals novel findings regarding ouabain’s sensitizing activity on TRAIL-induced apoptosis in lung cancer cells. The sensitizing effect of ouabain was found to be associated with the hydrogen peroxide-dependent degradation of Mcl-1 via the ubiquitin-proteasome pathway. We also showed for the first time that ouabain at physiological concentrations may play a role in enhancing the cancer cell response to TRAIL-induced apoptosis.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: P.C. and V.P. performed experiments; P.C. and V.P. analyzed data; P.C. and V.P. interpreted results of experiments; P.C. and V.P. drafted manuscript; P.C. and V.P. edited and revised manuscript; V.P. conceived and designed of research; V.P. prepared figures; V.P. approved final version of manuscript.

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